SIZE AND CONTRAST INCREASE THE DIVERTIVE EFFECT OF EYESPOTS

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RUNNING HEAD: Eyespots and attack diversion

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DATA ACCESSIBILITY

Analyses reported in this article can be reproduced using the data provided by Kjernsmo et al. 2018: doi:10.5061/dryad.h2q3d96

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ABSTRACT

Recent studies have shown that some eyespots of prey divert the strikes of predators, increasing the likelihood of prey escape. However, little is known about what makes eyespots effective divertive (deflective) prey marks. The size of eyespots varies much both between and even within taxa. Yet, whether size is important for the divertive function of eyespots is unknown. Furthermore, eyespots have often been described as highly contrasting, but the effects of contrast on the divertive function of eyespots has never been tested experimentally. Using artificial prey and the three-spined stickleback (*Gasterosteus aculeatus*) as a model for predator cognition and behaviour, we tested the importance of size as well as internal contrast for the divertive effect of eyespots. We independently increased the internal contrast and size of eyespots and found that both increased the divertive effect. The effect of size was significant over all four subsequent prey presentations, whereas the effect of contrast decreased after the initial presentations. These results suggest that the size and contrast of divertive marks are probably shaped by selection imposed by predation. We also discuss the involvement of predation in the seasonal and ontogenic plasticity of eyespots found in some taxa.

Keywords: diversion, deception, deflection, eyespot, *Gasterosteus aculeatus*, predation, protective coloration, visual attention

1. INTRODUCTION

Preventing detection is the first line of defence in many prey animals, but when disguise has been penetrated it is good to have another survival strategy at hand. One example of such strategy found in numerous prey, even many species that use camouflage for protection, are distinct marks that appear more conspicuous than the rest of the coloration. In many cases, such marks have been assumed to have a protective function, such that they divert an attacking predator by manipulating its attention and behaviour for the benefit of the prey (Poulton 1890, Cott 1940, Ruxton et al. 2004, Humphreys and Ruxton 2018). This strategy is based on the fact that, because of prey anatomy and escape behaviour, the consequences of predators' strikes directed towards different parts of the prey body are often not equally harmful to the prey. For this reason, it may pay for a detected prey to attempt to manipulate where an attacking predator will direct its strike. For example, the black tail tip of the white weasel is thought to have such a divertive function. Using models mimicking *Mustela frenata* and *M. erminea* and the red-tailed hawk (*Buteo jamaicensis*) as predator, Powell (1982) demonstrated that the black tail tips diverted the predator's attacks away from more vital parts of the weasel body.

A prominent type of colour pattern that has been suggested to divert (deflect) attacks by predators is the eyespot i.e. a colour pattern that consists of concentric rings of contrasting colours (Poulton 1890, Cott 1940, Blest 1957, Kodandaramaiah 2011, Humphreys and Ruxton 2018). Interestingly, eyespots are taxonomically widespread and occur for example in molluscs, insects, fishes, reptiles, birds, and mammals (e.g. Poulton 1890). Eyespots do not have the same function in all species, but several recent experimental studies show that eyespots of prey can manipulate where predators direct their strikes (Olofsson et al. 2010, 2013, Vallin et al. 2011, Kjernsmo and Merilaita 2013, Prudic et al. 2015, Kjernsmo et al.

2016). This effect is also supported by field studies. For example, Collins (2013) trapped 471 adult *Hyalophora* moths (fam. *Saturniidae*), recorded wing damage consistent with bird strikes and found that in a significant proportion (71%) of the cases the wing damage was located at an eyespot of the moth. Divertive effect induced by eyespots benefits the prey if, as a result of the diversion, the strike is aimed at a body part or direction that increases the chance that the prey survives an attack. Accordingly, the high proportion of strikes at the eyespot is likely to reflect the fact that in *Lepidoptera* wing tear is usually not fatal, hence a strike to the wing causes little damage compared to a strike to the body (Poulton 1890, van Someren 1922, Robbins 1980, Collins 2013, Ho et al. 2016, Humphreys and Ruxton 2018). It is also possible that eyespots, due to their location, draw strikes to a direction that makes it more likely that a predator completely misses the prey when it flees. For example, a posteriorly-located eyespot found in many species of fish, can increase the prey's chance of escape as it flees to the anterior direction. This may occur because the predator mistakes the posterior eyespot for the anterior, actual eye, and therefore moves in the opposite direction to the prey. Thus, the unexpected escape trajectory, due to the deception, could provide another explanation for the protective effect of a divertive eyespot (cf. Cott 1940, Robbins 1981, Wourms and Wasserman 1985, Kelley et al. 2013). Importantly, considering the evolution of eyespots, there is also direct experimental evidence that divertive eyespots increase prey survival (Olofsson et al. 2010, Prudic et al. 2015).

Despite the fact that attack diversion was proposed as one of the adaptive functions of eyespots already long ago (Poulton 1890), convincing experimental evidence for the divertive effect of eyespots has started to accumulate only recently (Olofsson et al. 2010, 2013, Vallin et al. 2011, Kjernsmo and Merilaita 2013, Prudic et al. 2015, Kjernsmo et al. 2016). Furthermore, although recent studies have provided experimental evidence for this function,

the optimisation of divertive eyespots has so far been studied little. Importantly, to increase our knowledge about how natural selection imposed by predation has shaped the specific appearance of protective prey marks, we must first seek to understand how their visual properties influence their function. For example, some previous studies suggest that the visual properties of eyespots influence predators' responses towards them. Olofsson and colleagues (2010) reported that eyespots in their experiment were divertive only under rather specific light conditions, more specifically under low light intensity with an accentuated UV-level. This was proposed to be attributable to a heightened contrast between the pupil of the eyespot and the surrounding area due to the specific light conditions (Olofsson et al. 2010), implying that contrast might influence the divertive potential of eyespots. An experiment by Prudic and colleagues (2015) suggests that a large and bright eyespot protects a butterfly (*Bicyclus anynana*) more effectively than small and dull eyespots against attacking mantids.

To better understand natural selection on divertive eyespots, it is necessary to study how the properties of eyespots influence predators' response towards them. For this reason, we wanted to separate size and internal contrast of eyespots to study if and how these two factors as such affect the divertive function of eyespots. Applying the setup used in (Kjernsmo and Merilaita 2013, Kjernsmo et al. 2016), we investigated the strikes of three-spined sticklebacks attacking artificial prey items that each had two different eyespots. Here we assume that not all parts of the prey items are equally vulnerable, and therefore manipulation of strikes towards certain direction will yield a survival benefit to the prey. In the first experiment, we tested whether the size of an eyespot influences the divertive effect. Due to its higher salience, we expected that a larger eyespot would attract a higher frequency of attacks towards it compared to a smaller eyespot. In the second experiment, we tested the effect of the contrast within the eyespot on the divertive effect. For the latter, we needed to manipulate the contrast of the

eyespots without changing the average lightness of the eyespot or the prey item. As increased internal contrast makes a pattern stand out more from the rest of the prey, we expected that fish would more often attack towards an eyespot with high internal contrast compared to an eyespot with lower internal contrast.

2. METHODS

General Procedure

In two separate predation experiments, we tested for the significance of size and internal contrast on the efficacy of divertive eyespots. Following the experimental protocol of Kjernsmo and Merilaita (2013) and Kjernsmo et al. (2016), we trained laboratory-reared three-spined sticklebacks to attack artificial prey items, and later tested their behavioural response towards prey with eyespots that either differed in size or contrast. To be able to test the effect of contrast without any confounding difference in average luminance between the low-contrast and high-contrast eyespots, we approximated the luminance vision of the three-spined sticklebacks using a visual model (Vorobyev and Osorio 1998). For this modelling, we needed estimates of the sensitivity of the photoreceptor cells of the sticklebacks. Such estimates for the three-spined stickleback have earlier been presented by Pike et al. (2011). However, basing the computations on the equations presented by Govardovskii and colleagues (2000) would produce more accurate, updated estimates. Therefore, before the second experiment addressing the effect of contrast, we computed the updated estimates of the sensitivities of the photoreceptor cells of the three-spined sticklebacks.

The predators and general holding conditions

The three-spined stickleback is a nearly circumpolar species on the Northern Hemisphere, found in a wide range of different salinities (Sigler and Sigler 1987, Östlund-Nilsson et al.

2007). We chose to use the three-spined stickleback as the predator in this study because it is easy to rear and maintain in the laboratory and it is considered primarily to be a visual predator (Wootton 1976, Ohguchi 1978, Hart and Gill 1994) and essential information about its vision is available (Rowe et al. 2004, Novales Flamarique et al. 2013, Shao et al. 2014).

In nature, sticklebacks are frequently parasitized by the common tapeworm (*Schistocephalus solidus*). This parasite has been shown to affect sticklebacks' behaviour (Giles 1987, Lobue and Bell 1993, Blake et al. 2006). To ensure that the sticklebacks' behaviour wasn't affected by any parasites, we used an artificially fertilized laboratory reared and thus parasite-free population. This also rendered us with naïve fish, i.e. fish that have had no previous experience of for example predators and prey. All the behavioural responses the fish showed during their initial encounters with the different prey types in both experiments can therefore be considered as innate.

The parental generation of the sticklebacks was collected with a hand trawl near Tvärminne zoological station (University of Helsinki) on the south coast of Finland (59° 50' N, 23° 12' E) at two different occasions, in June 2011 and 2012. Artificial fertilization was conducted shortly after, in the fish laboratory at the Department of Biosciences, Åbo Akademi University, Turku. After hatching, the fry were moved to small, 10-l aquaria and were fed with live brine shrimp (*Artemia sp.*) three times daily. When the fry were approximately two months old, the diet was gradually changed to a twice a day feeding of artificial flake food and thawed red mosquito larvae (*Chironomidae sp.*). At approximately one month of age the fish were moved to bigger, 200-l holding tanks. The water temperature ranged between 15-19 °C due to weekly water changes, and the light-dark rhythm was 13:11 h. The experiments were performed between fall 2012 and spring 2013 with permission from the Regional State

Administrative Agency of Southern Finland (decision number PH981A). All the fish used in the experiments were between 6 and 14 months old. The sticklebacks used in the fall 2012 started to develop their nuptial colouration mid-experiment, and so we had to pause the experiment until our new lab population had come of age (six months, according to previous experience; Kjernsmo and Merilaita 2013, Kjernsmo et al. 2016). For this reason, only 40 out of 117 fishes that entered the training sessions for the two experiments completed both the training and experimental phase (n = 25 for experiment 1, and n = 15 for experiment 2).

General experimental conditions

We produced the patterns for the artificial prey and the presentation backgrounds (5 x 7 cm foraging plates, Fig. 1) using a purpose-written program in MATLAB v. R2011a (The MathWorks, Inc. Natick, MA) and then printed the patterns on water-resistant paper ('Rite in the Rain', J. L. Darling Corporation, Tacoma, WA) with a laser printer (HP LaserJet P4015x with 1200 dpi resolution). Underneath each prey item (measuring 25 × 7 mm) we attached a piece of metal string with double-sided tape and self-adhesive plastic foil. This prevented the prey from moving around. Underneath the artificial prey we hid two mosquito larvae as an edible component, one larva at each end of the prey. The purpose of the foraging plate was to facilitate the attacks by generating an association between the foraging plate and the presented prey and food reward. Also, the conspicuousness of the prey could be adjusted to a preferred level, rendering the prey neither too easy nor too hard to detect from the background.

The experiments were run in eight 30-l aquaria (l × w × h = 50 × 20 × 30 cm), divided into three zones: a start zone, middle zone, and foraging zone (Fig. 2). The start zone and middle zone were separated by an opaque PVC sheet. In the beginning of each trial, the stickleback was located in the start zone and the foraging plate with a prey item in the foraging zone (Fig.

2). A trial started when the divider was raised, allowing the fish to swim to the foraging zone. Before the experiment, each fish was trained so that they would associate the artificial prey with the edible component hidden underneath.

The bottoms of the aquaria were covered with brown, adhesive plastic film. Natural daylight mimicking fluorescent lamps (36W, BIO vital®, NARVA, www.narva-bel.de) were used to illuminate the aquaria. The water temperature was around 16-18°C and the light-dark rhythm 13:11 h. To minimize external disturbance, we hung black plastic curtains with 5 x 10 cm observation holes in front of the aquaria.

Experiment 1: divertive effect and eyespot size

In the first experiment, we wanted to investigate how the size of an eyespot influences its divertive effect. The foraging plate used in this experiment consisted of a mottled, black-and-white pattern with the proportion of black set to 46 % (Fig. 1). The background pattern of the prey was also mottled, but with the proportion of black set to only 23 %. The prey had a large eyespot located on one half, and a small eyespot on the other half of the prey. The area of the large eyespot, 13 mm^2, was twice as large as the area of the small eyespot, 6.5 mm^2. In both eyespots, the diameter of the black centre was half of the diameter of the whole eyespot (Fig. 1). The training prey were plain white, *i.e.* lacked any pattering, while the foraging plate was the same as the one used in the experimental phase.

Before entering the experiment, a fish had to complete four training steps, two per day, one in the morning and one in the afternoon. During the approximately five-hour brake between the morning and afternoon training steps, the sticklebacks were deprived of food to assure that the hunger level of the fish was regained. On the day before the training started, the fish was

moved to an experimental aquarium and left to acclimatize there overnight. During the acclimation phase, the fish was placed behind the opaque divider in the start zone, and we placed two larvae on top of the white training prey on top of the foraging plate in the foraging zone (i.e. at the opposite end of the aquarium). The divider was then removed so that the fish was free to swim around. Next morning, if the fish was located in the middle zone or the foraging, it was gently moved back into the start zone before the training started. In training steps one and two, a larva was hidden under the training prey, approximately half of it showing from under the prey. In step three, only approximately 2 mm of each larva was visible, and in step four two larvae were completely covered by the artificial prey. Each training session started when the divider was raised and ended when the fish attacked the prey or after a maximum of 30 min. After a fish had completed the fourth training step, it proceeded to the experiment.

During the training phase, a trainer fish was included in each aquarium in an attempt to increase success rate and speed of the training. For this purpose, the aquarium was divided into two halves with a transparent wall parallel to the long side of the aquarium. As trainer fish we used experienced fish that had been used previously in similar predation experiments, each trainer fish was presented with the same training step at the same time as the focal fish. After the training, the trainer fish was removed from the aquarium, and the focal fish were alone in the aquaria during the experimental trials. The procedure with the trainer fish was only used in experiment 1 as this method only increased the workload, but not the success rate or speed of training.

In each of the four experimental trials, two chironomid larvae, were placed under a prey item such that the larvae were completely covered, one under each half of the prey. The side of the

large eyespot (*i.e.* left or right half of the prey as seen by the fish from the start zone) was altered between each trial, the side in the first trial being randomized. The schedule of the trials was similar as during the training, two a day, one in the morning and one in the afternoon. The experiment started when the divider was raised, and ended when the fish attacked the prey, or after a maximum of 15 min. Fish that didn't attack the prey within 15 min were excluded from the experiment. The behaviour of the fish (time to attack and which side of the prey was attacked) was recorded in real time with the event-recording software J-Watcher (version 1.0, available at: http://www.jwatcher.ucla.edu).

Experiment 2: divertive effect and eyespot contrast

In the second experiment, we investigated the effect of internal contrast of eyespots on the strength of the divertive effect. To this end, we produced prey that had two eyespots on the opposite halves, a high-contrast eyespot and a low-contrast eyespot (Fig. 1). Both the eyespots had the same geometry as the smaller eyespot in the prey used in Experiment 1. To be able to appropriately manipulate the grey shades constituting the patterns, we modelled the vision of the three-spined stickleback (see below). That enabled us to estimate the relative luminance of the grey shades as three-spined sticklebacks perceive them to calibrate the patterns. Here, we describe the shades proportionally to the luminance of the white of the paper (i.e. without print) of which the prey and foraging plate were made. Thus 100 % of the white corresponds to the luminance of the paper without any print, and 0 % of the white corresponds to black print. As the background shade of the prey items we used 50 % of the white, meaning a medium grey shade that in sticklebacks' eyes deviated equally much from the 100 % and the 0 % shades. The eyespots consisted of a light and a dark shade such that they both deviated equally much in luminance from the medium grey (50 %) but to opposite directions. Thus, the high-contrast eyespot consisted of black (0 %) and white (100 %) whereas the low-contrast

eyespot consisted of dark (25 %) and light (75 %) grey, but the total areas of the two shades were equal within each eyespot and hence the average luminance of the two eyespots were identical.

The pattern of the foraging plate, on which the prey items were presented consisted of randomly distributed patches of the same four different grey tones that were used for the high-contrast and the low-contrast-eyespots (i.e., 0 %, 25 %, 75 % and 100 % of the luminance of the white paper; Fig. 1). The total proportions of the four shades were equal, which ensured that both eyespots matched (or deviated from) the background to the same degree.

To be able to produce the shades that corresponded to the perceived luminance differences described above, we conducted a calibration that rendered us with the printer's grey values that correspond to the desired luminance values. To that end, we first needed to acquire the photoreceptor sensitivities of the three-spined stickleback and then to model its achromatic vision. The computation of the absorbance spectra of the photoreceptor cells are presented in the Supplementary Material.

We used these absorbance spectra to model the photoreceptor sensitivities of the three-spined sticklebacks (Pike et al. 2011), and calibrated the grey shades of the pattern of the prey and the foraging plate, using similar method as in previous studies (Dimitrova et al. 2009, Stevens et al. 2006). We produced a nine-step grey scale gradient ranging from black to white print and applied the visual model to produce an estimate of the luminance of each shade as perceived by the sticklebacks (Supplementary Material). Because double-cones are assumed to be predominantly responsible for luminance vision in these fish (Novales Flamarique et al. 2013, I Novales Flamarique, personal communication) and we were mainly interested in

luminance contrast, we specifically estimated the quantum catches of the double-cones. We then computed a third degree polynomial fit for these quantum catches as a function of the corresponding grey scale intensity values. We used this function and reflectance spectrometry to find the shade that produced the intermediate (50 %) magnitude of quantum catches compared to the extremes defined by the black print (0 %) and the white, unprinted paper (100%). The same way we acquired the two shades that produced 25 % and 75 % quantum catches and deviated equally much from the intermediate 50 % shade. We then used these five shades to produce the prey and the backgrounds for the contrast experiment (Fig. 1).

Both the training and the experimental trials were carried out in the same manner as experiment 1, except that no trainer fish were used during the training, and therefore also the aquaria were not divided longitudinally. This was because the use of trainer fish in experiment 1 increased the workload without improving the success rate of the training, when compared to previous, similar experiments (Kjernsmo and Merilaita 2013, Kjernsmo et al. 2016).

Statistical analyses

We used a two-tailed, exact binomial test (function 'binom.test' in R, v. 3.2.3, R Development Core Team 2015) to analyse whether the observed frequency of attacks the fish directed towards each half of the prey deviated from the expected value of 50% in both experiments. As each fish received four prey presentations, and were thus tested repeatedly, we corrected the significance threshold, α, for multiple testing using the sequential Bonferroni test (Rice 1989). To test whether eyespots interferes with learnt improvement of attack performance, we used attack latency as the dependent variable and tested whether there was a significant decrease in prey attack time as the fish gained experience attacking the spotted prey. Since these data were not normally distributed, we used the non-parametric Friedman test to test for

any differences in attack latencies between trials within an experiment. We predicted that the attack latencies would decrease as the fish became familiar with the setup. All analyses were conducted using the statistical software R for Windows (v. 3.2.3, R Development Core Team 2015).

3. RESULTS

Experiment 1: size of the eyespots

In the first experiment, we tested if eyespot size influences the divertive effect. Most of the sticklebacks directed their first attacks at the larger eyespot in the first trial (binomial test: n = 25, P = 0.015, corrected α = 0.025; figure 3A). The same effect was also observed in the three subsequent trials, where most of the fish directed their attacks towards the larger eyespot than the other half of the prey (second trial, binomial test: n = 25, P = 0.004, corrected α = 0.016; third trial: n = 25, P = 0.043, corrected α = 0.05; fourth trial: n = 25, P < 0.001, corrected α = 0.0125; figure 3A). Nine out of the 25 fish consistently attacked the large eyespot in all four trials, whereas no fish consistently attacked the small eyespot. Overall, the attack latency decreased successively as the fish gained learning experience from trial 1 to 4 (Friedman test: χ^2 = 24.81, n = 25, P < 0.0001; figure 3B).

Experiment 2: high- vs. low-contrast eyespot

In the second experiment, we tested if the contrast within the eyespot influenced its divertive effect. More of the sticklebacks directed their attack towards the high-contrast eyespot than towards the low-contrast eyespot in the first trial (binomial test: n = 15, P = 0.001, corrected α = 0.0125; figure 4A). In the second trial, the difference was not significant after the sequential Bonferroni correction (binomial test: n = 15, P = 0.035, corrected α = 0.016). In the third and the fourth trial, there was no significant effect of the level of internal contrast (third trial: n =

15, $P = 0.61$, corrected $\alpha = 0.05$; fourth trial: $n = 15$, $P = 0.302$, corrected $\alpha = 0.025$; figure 4A). Six out of the 15 fish consistently attacked the high contrast eyespot in all four trials, whereas no fish consistently attacked the low contrast eyespot. Attack latency of the fish did not differ significantly between the four trials (Friedman test: $\chi^2 = 2.44$, $n = 15$, $P = 0.486$; figure 4B).

4. DISCUSSION

Here, we have demonstrated that visual properties of eyespots are important for their divertive effect. An increase in size and internal contrast both enhanced predator diversion, as the majority of the fish directed their first strike towards the larger spot in experiment 1 and the more contrasting eyespot in experiment 2. Because we know that these fish were naïve to prey with eyespots prior to these experiments, we could consider at least their first encounter with the spotted prey as an innate response towards these markings (cf. Kjernsmo and Merilaita 2013, Kjernsmo et al. 2016).

Our results show that eyespots effectively draw the strikes of attacking fish towards them, providing empirical support for the idea that eyespots have an anti-predator function by means of diversion (Olofsson et al. 2010, Merilaita et al. 2011, Kjernsmo and Merilaita 2013, Olofsson et al. 2013, Prudic et al. 2015, Kjernsmo et al. 2016). Moreover, our experiments clearly demonstrate that the attack behaviour of visual predators can be markedly manipulated by altering the visual properties of prey markings, and that it only takes a relatively small adjustment of the eyespot pattern to influence predators' response. Jointly, our results suggest that the divertive effect of eyespots and possibly other colour markings can be strengthened by increasing the size or the internal contrast of the marking. This is important when natural selection on divertive eyespots is considered. Interestingly, the divertive effect decreased with

increased number of trials in Experiment 2 (contrast), but not in Experiment 1 (size). This suggests that the fish became habituated to increased contrast of the experimental eyespots, whereas for increased size such habituation did not occur, and the divertive effect of the larger eyespot remained strong across all trials. Another difference was that in Experiment 1 attack latency decreased towards the last trial, but not in Experiment 2. However, comparison between the results of the two experiments is complicated, in addition to the difference in the number of replicates, by the deviating size of the large eyespot in Experiment 1 and by the possibility that the effect of eyespot contrast may interact with the effect of size. For this reason, the result does not enable conclusions regarding the relative importance of the two.

Our findings are in accordance with a recent study testing for the divertive effect of eyespots located at the wing margins of the squinting bush brown butterfly, *Bicyclus anynana* (Prudic et al. 2015). *B. anynana* has two visually distinct phenotypes, the wet season morph with large, high-contrast eyespots, and the dry season morph with reduced, dull eyespots. Prudic et al. (2015) presented both morphs to praying mantids (a natural predator of *B. anynana*) and measured the attack frequency and reproductive success of the butterflies. They found that butterflies with larger, more contrasting eyespots were struck on the eyespot more frequently, but importantly also managed to escape the attacks more often, resulting in a higher reproductive success and fitness advantage for them (Prudic et al. 2015). The authors suggest that the increase in predator abundance during the wet season may have driven the evolution of seasonal polyphenism in the expression of the eyespot in these butterflies (Prudic et al. 2015). However, it is noteworthy that in their study eyespot size and contrast did not vary independently, and interestingly, our present study shows that both these properties can separately influence the divertive effect of eyespots.

Eyespot plasticity is also found in many species of fish, and for example in several butterflyfishes and damselfishes, the adults lose the posteriorly located eyespot expressed in juveniles (Gagliano 2008, Kelley et al. 2013). As in the seasonally polyphenic butterflies, also such ontogenic plasticity may have evolved as a response to variation in predation risk. It is noteworthy, that each prey item in the present study had two visually distinct eyespots located in each end on the prey "body". This made it possible to study the direct effects of difference in size and internal contrast on the attack behaviour of the predators when two prey marks are competing for the visual attention of the predator. Because it is in a predator's interest to swiftly immobilize its prey, a strike towards the head of an animal would seem like an obvious and effective way to accomplish success of the attack. It is therefore likely that many predators use simple cues, such as the eye, to locate and target a prey's head (Cott 1940, Wickler 1968, Zaret and Kerfoot 1975, Wourms and Wasserman 1985). In addition, the location of the eye can also provide a predator with information about the escape trajectory of the prey. Thus, successful diversion of a predator may require that an eyespot needs to outcompete other salient features of the prey body, such as the actual eye, in terms of visual attention of a predator. Our present experiments suggest that in such cases of deception selection on size and contrast in relation to those of the actual eye has an important role in the evolution of the eyespot. Indeed, this seems to be the case in many species of fish that sport eyespots (Gagliano 2008, Kelley et al. 2013). For example, in a comparative study on the butterflyfishes (fam. *Chaetodontidae*), Kelley et al. (2013) found that in all species that had eyespots located close to the caudal fin area, the "pupil" of the eyespot was larger than the pupil of the fish's real eye and also darker than their background skin colour. The relative salience of the real eye can further be decreased by an eye stripe found in many species (Kjernsmo et al. 2016). Thus, when considering the evolution of the whole colour pattern of a prey that sports divertive marks, we would expect that three important factors influence each

other: the relative salience of the divertive marks, concealment of salient features and the overall need for camouflage (Kjernsmo et al. 2016).

Our finding that larger eyespots are more effective at diverting attacks by predators is interesting also when considering the anti-predator functions of eyespots. It has commonly been assumed that (relatively) large eyespots have a startling effect on predators, such that they make a predator hesitate or halt its attack, whereas smaller eyespots are more likely to serve a divertive function (Stevens 2005, Vallin et al. 2005, Kodandaramaiah et al. 2009, Olofsson et al. 2012, Kodandaramaiah et al. 2013). However, these two functions (predator diversion and startling of predators) may not be mutually exclusive, which would be important when the evolutionary dynamics of the eyespot pattern is considered. For example, the size of the predator may be an important factor determining its response towards an eyespot. Another question for future studies is whether some diverting eyespots benefit from eye mimicry. Eye mimicry has been shown to underlie the startling effect of eyespots (Kjernsmo and Merilaita 2017), but it is possible that eye mimicry might enhance the divertive effect of eyespots as well. For example, eye mimicry could contribute to the diversion generated by the posterior eyespots found in many fishes (Kelley et al. 2013) and give a false idea of the location of the head and the escape trajectory of the prey (Kjernsmo et al. 2016).

To conclude, our results show that both size and contrast, independently from each other, have the potential to influence the anti-predator efficacy of eyespots by altering the attack behaviour of visually hunting predators. Thus, an increase in either or both of these properties can significantly increase the divertive effect of eyespots, which is pivotal for their protective

function. Ultimately, these findings shed light not only on the existence of eyespots in many species of prey, but also on their appearance.

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6. FIGURE LEGENDS

Figure 1. A) The prey item (25 x 7 mm) with a large and a small eyespot and B) the background (foraging plate, 5 x 7 cm) that were used in experiment 1 to test the effect of eyespot size on attack diversion. C) The prey item with a low contrast and a high contrast eyespot and D) the background, on which both eyespots appeared equally conspicuous, used in experiment 2 to test the effect of internal contrast of the eyespot on the divertive effect of eyespots.

Figure 2. A schematic of the experimental setup. Each fish was initially located in the start zone, which was separated from the middle zone by an opaque plastic divider. The prey item, covering two pieces of mosquito larvae as a food reward, was located on top of the foraging plate in the foraging zone.

3. Results from experiment 1 (large vs. small eyespot). A) Frequency of attacks the fish directed towards the two halves of the prey during trial 1 to 4. Number of attacks directed at the large eyespot (black bars) and the small eyespot (white bars). B) Median attack latencies ± the interquartile range in seconds in trials 1–4.

Figure 4. Results from experiment 2 (high- vs. low-contrast eyespot). A) Frequency of attacks the fish directed towards the two halves of the prey during trial 1 to 4. Number of attacks directed at the high-contrast eyespot (black bars) and the low-contrast eyespot (white bars). B) Median attack latencies ± the interquartile range in seconds in trials 1–4.